EVALUATION OF LABORATORY METHODS FOR SUSCEPTIBILITY TESTING OF STAPHYLOCOCCUS AUREUS.

Hermanus Christoffel Jansen van Rensburg

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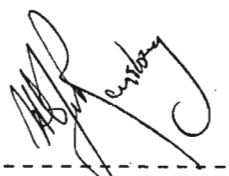
DECLARATION

I declare that this is my own work.

This project has not been submitted before for any degree or examination in any other university.



H.C. JANSEN VAN RENSBURG

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ABSTRACT

The susceptibility of 80 Staphylococcus aureus isolates to oxacillin was investigated using microtitre, agar dilution and Stokes' disc diffusion methods. There was a bimodal distribution of the isolates according to the oxacillin minimum inhibitory concentration (MIC) values. For the sensitive isolates, the agar dilution method generally gave lower MIC values than the microtitre method, while for the resistant isolates the agar dilution method gave comparable to slightly lower MIC values than the microtitre method. The Stokes disc diffusion method yielding the best results when performed on Mueller-Hinton agar incubated at 30°C for 18 hours; however local strains grew poorly when incubated at 30°C for 18 hours. The next best medium which provided clear disc diffusion results plus good growth was Mueller-Hinton agar incubated at 35°C for 18 hours, on which 10 % of the sensitive isolates appeared intermediate in susceptibility, and none resistant, while all the resistant isolates (microtitre MIC ≥8mg/l) appeared resistant. Oxacillin resistance among strains of Staphylococcus aureus tested by Stokes' disc diffusion method correlated best with gentamicin resistance, and less often with tetracycline resistance. Therefore gentamicin- or tetracycline-resistance may indicate oxacillin resistance in Staphylococcus aureus.

INTRODUCTION

OVERALL IMPORTANCE

Since Alexander Ogston's original description of Staphylococcus aureus (S. aureus), this versatile bacterium has remained an important cause of both hospital- and community-acquired infections (1). The spectrum of infections caused by S. aureus is wide and may range from mild, self-limiting, to serious and life-threatening in severity, and from superficial to deep and disseminated in extent.

Little has been published in respect of S. aureus infections in South Africa. In a recent publication from Tygerberg Hospital, 35% of all S. aureus bacteraemias were found to be community acquired. Osteitis and septic arthritis were the most common serious systemic diseases, being present in 46% of all community-acquired S. aureus bacteraemias (2).

At Tygerberg hospital, septicaemia (31%) and wound sepsis (29%) were the most common conditions in nosocomially acquired bacteraemia (2).

In an analysis of blood culture isolates from 7 South African teaching hospital centres, van den Ende and Rotter showed that for 1983, S. aureus was the most common blood culture isolate in 5

centres. At Ga-Rankuwa it ranked second, and in Durban it ranked fourth. In 1984 there was a significant overall decrease in frequency of S. aureus isolations, with it ranking first in only 3 centres, namely Johannesburg, Ga-Rankuwa and Cape Town (3).

At King Edward VIII Hospital, S. aureus was the third commonest (13%) blood culture isolate in 1986; in 1987, S. aureus was the commonest (17%) blood culture isolate.

TREATMENT OF S. AUREUS INFECTIONS AND DEVELOPMENT OF RESISTANCE

The antimicrobial resistance patterns for S. aureus have changed in the last four decades, and consequently many strains now exhibit resistance to antimicribial agents of choice, and in some cases have become multi-resistant.

Before the introduction of penicillin in the early 1940's the prognosis for patients with serious S. aureus infection was often very poor. The introduction of penicillin for the treatment of these infections led to marked reduction in morbidity and mortality (4). However, soon after the introduction of penicillin, an increasing incidence of penicillin resistance in S. aureus strains was reported (5), and by 1948 up to 60% of hospital strains of S. aureus were penicillin-resistant (6).

By the end of the 1950's, at the University hospital in Seattle, U.S.A., at least 85% of S. aureus strains were resistant to penicillin and streptomycin, 60% were resistant to

tetracycline, 43% resistant to erythromycin and 28% resistant to chloramphenicol (7).

In the early 1960's methicillin, the first semi-synthetic penicillin resistant to the hydrolysis by staphylococcal beta-lactamase, was introduced (8). The therapeutic problems caused by the increase in beta-lactamase producing, and therefore penicillin-resistant and often multi-resistant S. aureus strains, were alleviated with the introduction of methicillin and other members of this group of penicillinase-resistant penicillins (PRP) which includes oxacillin, nafcillin and cloxacillin (4). These agents soon became the mainstay of therapy for S. aureus infection. Not long thereafter - during the 1960's - some European countries reported the emergence and increase of methicillin resistance in S. aureus.

Reports from Switzerland described an increase in methicillin resistance among S. aureus isolates from 9.7 percent in 1965 to 17.3 percent in 1966 (9).

British hospitals also witnessed an increase in methicillin-resistant staphylococcus strains during the 1960's, with prevalences increasing from about 1 percent in 1965 to 5 percent in 1969 in the London area (10).

In the USA, a fall in the incidence of multiple antibiotic resistance in S. aureus strains in the University of Washington

Hospital, Seattle, was reported. Over 40 percent of staphylococci were resistant to four or more antibiotics in 1959 but by 1967 this proportion had fallen to less than 10 percent (7).

In 1980 workers at Austin Hospital in Australia showed that 32% of all S. aureus isolates were methicillin-resistant. Their data revealed that methicillin-resistant S. aureus strains were an uncommon cause of infection in the general community. They also found that factors that predisposed to infection or colonisation with these methicillin-resistant strains were old age and prolonged hospitalisation (11).

Significant methicillin resistance among S. aureus, especially among hospital isolates, has been recognized in South Africa since the late 1970's. Overall methicillin resistance rose from 28.7% in 1983 to 33.9% in the 1984 period. From 1983 to 1984 there was a significant increase in methicillin resistance from 31.9% to 50.8% amongst S. aureus isolates in Johannseburg , whilst significant decreases from 52.7% to 39.6% for Pretoria and from 17% to 4.6% for Tygerberg hospital were noted. In 1984 in Durban, 10.5% of S. aureus isolates were methicillin-resistant. At King Edward VIII Hospital, the prevalences of methicillin-resistant S. aureus isolates from blood cultures were 32% and 29% for 1986 and 1987 respectively (data not published). At Tygerberg hospital all community-acquired S. aureus bacteraemias were methicillin-sensitive, whilst 48% of hospital-acquired S. aureus bacteraemias

were methicillin-resistant (2).

ANTIBIOTIC SUSCEPTIBILITY TESTING

Accurate, reproducible antibiotic susceptibility testing is of utmost importance not only for treating individual patients with serious bacterial infections; but also, when analysed collectively serves as a basis for formulating an antibiotic policy or for epidemiological purposes.

The susceptibility testing of S. aureus against the penicillinase-resistant penicillins (PRP) remains a problem. Methicillin-resistant staphylococci may be either heterogenous or homogenous in their expression of resistance (12). The strains that appear heterogenous consist of a majority population with a comparatively slight increase in resistance and a minority population with a considerable increase in resistance (13,14). In contrast, strains exhibiting homogenous resistance consist of a population expressing uniform resistance. The phenotypic expression of heteroresistance may be influenced by various in-vitro conditions such as incubation temperature, salt (NaCl) concentration, pH, duration of incubation, and inoculum size.

Annear (15) reported that methicillin-resistant cultures showed enhanced resistance when grown on ordinary nutrient medium at 31° C, and that the temperature of incubation had little effect on the susceptible isolates. This trend was demonstrated with the

broth dilution, agar dilution and disk diffusion methods of suseptibilty testing.

Milne et al (16) in their comparative study of culture media for detecting methicillin resistance in S. aureus by the agar dilution method, reported that Sensitest and Iso-Sensitest media performed less well than Mueller-Hinton, Columbia and Diagnostic Sensitivity Test (DST) agar after 18 hours incubation. After 40 hours incubation Mueller-Hinton agar with 5% added sodium chloride detected 100% of resistant strains whilst Columbia and DST with 5% added NaCl detected 84%. Even the source and composition of Mueller-Hinton agar may influence the susceptibility testing of S. aureus (17).

Barber (18) showed that by adding excess electrolytes (5% NaCl or 7,5% $(NH4)_2 SO_4$) or by decreasing the agar concentration, growth of S. aureus in the presence of methicillin was almost equal to that on control plates.

Churcher (19) reported that it was possible to divide staphylococci, which showed a reduced zone of inhibition on routine sensitivity agar, into methicillin-resistant and - sensitive group, when tested on agar containing 5% NaCl.

Sabbath and Wallace (20) noted that S. aureus resistance was masked in an acid medium and that there was a marked slower growth rate in an acid medium when the methicillin resistance decreased

to levels seen in sensitive strains.

Prolonged incubation increased the detection of PRP-resistant S. aureus (14). However, Milligan et al (21) noted that when the disc diffusion test was incubated for 48 hours it could lead to a high degree of ambiguous or inaccurate results and concluded that disc diffusion tests should not be incubated for longer than 24 hours.

Sutherland and Rolison (14) further noted that the inoculum size had a significant effect on the zone size when the disc diffusion method was used.

Methods used for determining the in vitro susceptibility of staphylococci include broth microdilution, broth macrodilution, agar dilution and disc diffusion.

The broth microdilution method was extensively evaluated by Thornsberry and McDougal (22) who made the following recommendations, namely:

(i) use Mueller-Hinton broth supplemented with calcium and magnesium as the basic medium for performing these tests, as recommended by the NCCLS (23), and add 2% NaCl to the broth that will be used for testing methicillin, oxacillin, nafcillin, or cephalothin.

(ii) perform test as described in NCCLS standard M7-T (23), but with preparation of the inoculum from growth off an

overnight agar plate and incubate the trays or tubes at 35°C for a full 24 hours.

(iii) record the MIC as the lowest concentration that inhibits macroscopic growth.

Barry and Jones (24) showed that microdilution tests with oxacillin in broth with 2% NaCl were more reliable than similar tests with methicillin.

The agar dilution method is mainly used as a screening method.

Thornsberry and McDougal (22,25) recommend the use of Mueller-Hinton agar containing 4% NaCl and either 10 mg/l of methicillin, 6 mg/l of oxacillin or 6 mg/l of nafcillin. Milne et al (16) recommend Mueller-Hinton agar with 5% added NaCl incubated at 35°C for 40 hours as the agar dilution method for testing S. aureus resistance to methicillin.

In 1972 Drew et al (26) concluded that the standard disc test (Kirby-Bauer) was satisfactory for identifying most PRP-resistant staphylococci and recommended the use of an oxacillin disc rather than methicillin because the latter was found to be less stable during storage. They claimed, however, that 1 ug cloxacillin discs failed to discriminate between sensitive and resistant staphylococci by zone measurement.

In 1984 Boyce (27) recommended the use of a 1 ug oxacillin disc in preference to the 5 ug methicillin disc and demonstrated that

the disc diffusion test (Kirby-Bauer) must be incubated at 35°C for a full 24 hours, rather than the usual 16 to 18 hours, to obtain reliable results. In 1984 McDougal and Thornsberry (28) recommended that oxacillin (4ug) be the antibiotic of choice to represent the PRP for the Kirby-Bauer disk diffusion susceptibility test for methicillin-resistant (heteroresistant) staphylococci, and recommended new zone and MIC breakpoints. In contrast, Barry and Jones (24) concluded that the standardized disc suseptibility test (Kirby-Bauer) with 1 ug oxacillin discs, was reasonably reliable for detecting staphylococcal resistance, provided that the plates were incubated for a full 24 hours at 35°C and that the inhibition zones were carefully examined to detect smaller colonies.

Oxacillin is the antibiotic of choice for determining the susceptibility of S. aureus to the PRP because it is more stable during storage (26) than the other PRP's. It, however, is more susceptible to staphylococcal β-lactamase than the other PRP's (25,29).

At King Edward VIII Hospital Microbiology Laboratory, the routine method for oxacillin susceptibility testing of S. aureus is the Stokes method using Iso-Sensitest agar incubated at 30°C for 16-18 hours. An unacceptably high incidence of apparent intermediate sensitivity was encountered. It has been noted locally that patients with osteomyelitis, caused by S. aureus with

intermediate sensitivity to oxacillin, responded clinically to cloxacillin treatment.

This study was undertaken to determine the true susceptibility of local isolates to penicillinase-resistant penicillins (PRP) and to evaluate the different susceptibility testing methods in order to determine the most reliable method for local laboratory use. Resistance to other antimicrobial agents was also evaluated as potential indicator(s) that the isolates may be resistant to the PRP's.

MATERIALS AND METHODS

BACTERIAL STRAINS

The 80 strains of S. aureus included in this study were obtained from specimens submitted to the Microbiology Laboratory at King Edward VIII Hospital over the period August 1987 to August 1988. Strains were chosen on the basis of disc diffusion susceptibility testing results obtained in the routine laboratory. Twenty strains were sensitive, 40 intermediate, and 20 resistant. Most isolates were from blood cultures. The identity of these isolates were established by Gram stain, catalase and the DNase results.

MEDIA

Columbia, Diagnostic Sensitivity test (DST), Iso-Sensitest and Mueller-Hinton media (Oxoid Ltd, Basingstoke, Hants, England) were used in the susceptibility evaluations.

The medium for the microdilution method was prepared as recommended by Thornsberry and McDougal (22). Mueller-Hinton broth was supplemented with calcium, magnesium and NaCl to give a final concentration of 50mg/l calcium, 25mg/l magnesium (CSMHB) and 2% NaCl.

Media for the agar dilution method were prepared according to the manufacturers' instructions, except that the initial suspensions of the media powders were made in eight tenths of the recommended

volumes of water. After sterilizing and cooling to 45°C the remaining two tenths of the volume was made up by the addition of appropriate sterile aqueous solutions of antimicrobial agents.

Media for the disc-diffusion tests were prepared according to the manufacturers' instructions.

ANTIBIOTICS

For the broth microdilution method and agar dilution method, reference antibiotic materials were obtained from the appropriate pharmaceutical companies.

The antimicrobial agents used in the microtitre method were:

penicillin (Beecham Pharmaceuticals, U.K.)

oxacillin (Beecham Pharmaceuticals, U.K.)

cephalothin (Eli Lilly and Company, U.S.A.)

The concentrations for oxacillin and cephalothin ranged from 128 mg/l to 0,12 mg/l. For penicillin, the concentrations ranged from 64 mg/l to 0.06 mg/l.

The microtitre trays were kept at -20°C and used within 2 weeks of preparation.

The antimicrobial agents used in the agar dilution method were the same as in the microtitre method except that the concentrations ranged from 128 mg/l to 0.007 mg/l.
The plates were kept at 4°C and used within 24 hours of preparation.

For the disc diffusion tests, antibiotic discs were obtained from Mast Laboratories (Liverpool, U.K.). Mast ring named GP 1 contained the following six antibiotics ; penicillin (1ug), erythromycin (10ug), tetracycline (10ug), oxacillin (1ug), chloramphenicol (10ug) and cephalothin (30ug).
Mast ring named GP2 contained the following antibiotics ; clindamycin (2ug), gentamicin (10ug), fusidic acid (10ug), cotrimoxazole (25ug), rifampicin (5ug) and vancomycin (30ug).

METHODS

Microdilution method:

The microdilution method was performed as described in NCCLS standard M7-T (23) exept for variations in media, incubation and inoculum preparation.

The following recommendations of Thornsberry and McDougal (22) were followed, namely:

(i) CSMH broth as the basic medium as recommended by NCCLS (23) with 2% NaCl added to the broth ;

(ii) the trays were incubated at 35°C for a full 24h ;

(iii) the MIC was recorded as the lowest concentration that inhibited macroscopic growth.

The inoculum was prepared by inoculating CSMH broth containing 2% NaCl with 1 colony of an overnight culture, incubating it at 35°C for 2-4 hours, and then standardizing the turbidity to 0,5 McFarland standard containing approximately 10^8 cfu/ml. The

final inoculum was approximately 2.5 x 10^5 cfu/ ml.

Agar dilution method:

The agar dilution method was performed as described in NCCLS standard M7-T (23), except for variations in media, incubation and inoculum preparation.

The antibiotics tested were penicillin, oxacillin and cephalothin on Mueller-Hinton agar containing 4% NaCl.

In addition oxacillin was tested on Mueller-Hinton agar not containing NaCl and on DST and Columbia agar with and without 4% NaCl. The plates with NaCl were incubated at 35°C and those without NaCl at 30°C.

The inoculum was prepared as in the microdilution method except that the broth was nutrient broth. The plates were inoculated with a multipoint inoculator, ie. 0,1ul of a 10^8 cfu/ml inoculum to give a final inoculum of approximately 10^4 cfu/spot (30).

The MIC was recorded after 18, 24 and 48 hours of incubation as the lowest concentration at which macroscopic growth was inhibited.

Disc diffusion method:

Disc diffusion tests were performed according to the method of Stokes (31), except that the inocula were prepared in distilled water instead of broth.

Columbia, DST, and Mueller-Hinton agar were used under the

following three conditions, namely : (i) without NaCl at 30°C; (ii) without NaCl at 35°C; and (iii) with 4% NaCl at 35°C.

Iso-Sensitest agar with antibiotics GP1, which included the 1ug oxacillin disc, were incubated at 30°C. The plates were read after 18, 24 and 48 hours of incubation.

INTERPRETIVE CRITERIA

Microtitre and agar dilution methods:

The sensitive and resistant breakpoints were $\leq$2 and $\geq$8 mg/l for oxacillin, and $\leq$8 and $\geq$32 mg/l for cephalothin, respectively.

Stokes disc diffusion method:

Sensitive	:	inhibition zone size (radius) equal to, greater than, or not more than 3 mm smaller than that of the control
Intermediate	:	inhibition zone size (radius) greater than 3 mm, but 3 mm smaller than that of the control
Resistant	:	inhibition zone size (radius) 3 mm or less, or absent

The Oxford strain of S. aureus NCTC 6571, which is fully sensitive to the PRP, was the control strain used in all tests.

RESULTS

OXACILLIN SUSCEPTIBILITY TESTING

MICROTITRE METHOD

The distribution of the 80 S. aureus isolates according to microtitre MIC for oxacillin is shown in figure 1. The isolates fell into two distinct groups, namely sensitive and resistant. The majority of the 60 sensitive isolates had a MIC of 1 mg/l, while the majority of the 20 resistant isolates had a MIC of 64 mg/l. There were no isolates with MIC's of 4 mg/l, which would have been indicative of intermediate susceptibility. The microtitre method was used as reference method.

AGAR DILUTION METHOD

The agar dilution method generally gave lower MIC 50, MIC 90 and mean MIC values than the microtitre method, as depicted in table I. On evaluation of the different agar dilution methods, it was found that Mueller-Hinton agar, read at 24 hours, gave lower MIC values than the microtitre method for the sensitive strains, while for the resistant isolates the MIC values were comparable to,or even higher than those obtained with the agar dilution method (figure 2).

DST agar, incubated for 24 hours, had lower MIC values than the microtitre method, for all isolates. The bimodal distribution of the isolates was also less pronounced (figure 3).

Columbia agar, incubated for 24 hours, yielded lower MIC values for the sensitive strains. For the resistant isolates, the MIC values obtained by the microtitre and agar dilution methods were comparable. The bimodal distribution of the isolates was more pronounced, especially with Columbia agar incubated at 30°C (figure 4).

DISC DIFFUSION METHOD

Mueller-Hinton agar:

After 18 hours of incubation, none of the sensitive isolates (MIC $\leq$2mg/l) yielded an intermediate result on Mueller-Hinton agar at 30°C, while 8 isolates gave an intermediate susceptibility result on Mueller-Hinton agar incubated at 35°C, and 19 on Mueller-Hinton agar with 4% NaCl incubated at 35°C.

Proportionately more isolates yielded intermediate susceptibility and resistance after 24 and 48 hours of incubation. Mueller-Hinton agar with 4% NaCl incubated at 35°C for 48 hours yielded the highest number of resistant isolates. Under all conditions, all isolates with a microtitre MIC $\geq$8mg/l showed resistance by the disc diffusion method (table II).

Diagnostic sensitivity test agar (DST):

Some isolates with MIC's $\leq$2mg/l appeared intermediate on all 3 methods after 18 hours of incubation. The media showing the least number of isolates with intermediate sensitivity was DST

incubated at 30°C. Proportionately more isolates showed intermediate susceptibility or resistance after prolonged incubation. The highest number showing resistance was on DST agar with 4% NaCl (DSTS) incubated at 35°C for 48 hours.

Some of the isolates with an oxacillin microtitre MIC ≥8 mg/l appeared intermediate to oxacillin by the disk diffusion method. On DST at 30°C, 2 isolates appeared intermediately susceptible after 18 hours incubation while all isolates were resistant after 24 and 48 hours of incubation.
Of the isolates with a MIC of ≥8 mg/l, none showed intermediate susceptibilitty on DST incubated at 35°C for 48 hours while 2 isolates showed intermediate susceptibility on DST agar with 4% NaCl incubated at 35°C for 48 hours (table III).

Columbia agar:
After 18 hours incubation 2 isolates with MIC's of ≤2mg/l appeared intermediately susceptible on Columbia agar at 30°C, while 12 and 26 isolates respectively showed intermediate susceptibility on Columbia agar at 35°C and Columbia agar with 4 % NaCl incubated at 35°C.

Some of the isolates with a MIC ≥8 mg/l showed intermediate susceptibility to oxacillin by the disk diffusion method. After 48 hours of incubation no isolates showed intermediate susceptibility on Columbia agar at 30°C or Columbia agar with 4 % NaCl at 35°C, whilst 1 isolate showed intermediate susceptibility

on Columbia agar at 35°C (table IV).

Iso-Sensitest agar:

Of the 60 sensitive isolates (MIC ≤2 mg/l) 14 showed an intermediate susceptibility after 18 hours of incubation and proportionately more after 24 and 48 hours of incubation. Of the 20 resistant isolates 2 had an intermediate susceptibility after 18 hours, 1 after 24 hours, and none after 48 hours of incubation (table V).

CEPHALOTHIN SUSCEPTIBILITY TESTING

Microtitre method:

In figure 5 the distribution of the isolates is shown. Sixty four isolates had a MIC of ≤8mg/l, 11 a MIC of 16mg/l, and 5 a MIC of ≥32mg/l.

Agar dilution method:

In figure 6 the comparison is shown between the microtitre and agar dilution methods. For the sensitive strains the agar dilution method gave lower MIC values than the microtitre method, while for the resistant strains the MIC values were comparable to slightly higher.

Disc diffusion method:

The results are shown in table VII.

When the disc method was performed according to the Stokes method on Mueller-Hinton agar incubated at 35°C for 18 hours, 56 of the

64 sensitive isolates (MIC $\leqslant$8 mg/l) appeared sensitive, 8 were intermediately susceptible and none appeared resistant. Of the 11 isolates with an intermediate susceptibility (MIC = 16mg/l), 7 showed intermediate susceptibility and 4 appeared resistant. Of the 5 resistant isolates (MIC $\geqslant$32 mg/l), 3 showed intermediate susceptibility and 2 appeared resistant.

RELATIONSHIP BETWEEN OXACILLIN MIC AND CEPHALOTHIN MIC.

Of the 20 oxacillin resistant isolates (microtitre MIC $\geqslant$8 mg/l), 4 were sensitive (MIC $\leqslant$8mg/l) and 11 were of intermediate susceptibility (MIC = 16 mg/l) to cephalothin. The remaining 5 were fully resistant to cephalothin (microtitre MIC $\geqslant$32), as shown in table VI.

ASSOCIATION BETWEEN OXACILLIN SUSCEPTIBILITY AND SUSCEPTIBILITY TO OTHER ANTIMICROBIAL AGENTS

Thornsberry and McDougal (22) noted that multiple resistance in a S. aureus strain could be an indicator of the possibility of heteroresistance to oxacillin in that particular strain. Of the 60 oxacillin-sensitive isolates (microtitre MIC $\leqslant$2 mg/l), 8 (13%) were sensitive to penicillin, 56 (93%) to erythromycin, 59 (98%) to tetracycline, 59 (98%) to chloramphenicol, 55 (91%) to cephalothin, 60 (100%) to clindamicin, and 60 (100%) to gentamicin, as shown in table VIII.

Of the 20 oxacillin resistant isolates (microtitre MIC $\geqslant$8mg/l), 6

(30%) were resistant to erythromycin, 10 (50%) to tetracycline, 4 (20%) to chloramphenicol, 6 (30%) to cephalothin, 1 (5%) to clindamycin, and 16 (80%) to gentamicin.

DISCUSSION

The accurate detection of oxacillin resistant S. aureus has important clinical and epidemiological implications. Failure to detect resistant organisms by susceptibility tests leads to a false sense of security or inadequate therapy of serious S. aureus infections. On the other hand, false reporting of resistance could lead to the unnecessary, although well intended, use of alternative inappropriate antimicrobial agents, which may be toxic. Correct reliable susceptibility testing is therefore of utmost importance.

OXACILLIN MICROTITRE METHOD

The S. aureus isolates in this study fell into distinct groups according to oxacillin microtitre MIC's. The majority of the sensitive isolates had a MIC of 1 mg/l while the majority of the resistant strains had a MIC of 64mg/l. There were no isolates with intermediate susceptibility (MIC = 4 mg/l).

OXACILLIN AGAR DILUTION METHOD

All strains of S. aureus gave visible growth on each medium tested after 18 hours of incubation.

Sensitive isolates:

ST agar with 4 % NaCl gave the lowest MIC values, followed by olumbia and Mueller Hinton agar. All agar dilution methods gave

lower overall MIC values than the microtitre method.

Resistant isolates:

In their evaluation of culture media for detecting methicillin-resistance by the agar dilution method, Milne et al (16) found that Sensitest and Iso-sensitest media performed less well than Mueller-Hinton, DST and Columbia agar. After 40 hours incubation Mueller-Hinton agar with 5% added NaCl detected 100% of the strains, and Columbia and DST with 5% added NaCl detected 84%. After 40 hours the performance of Mueller-Hinton agar, DST, and Columbia agar, incubated at 30° C, was similar in that they detected 87%, 90% and 81% respectively of the resistant strains. In this study oxacillin was used as antibiotic to determine resistance to the penicillinase-resistant penicillins (PRP), whilst Milne at al used methicillin. In this study 4% NaCl was added to the media whilst Milne et al added 5% NaCl (16).

Milne et al recommend Mueller-Hinton agar with 5% NaCl incubated at 35° C for 18 - 40 hours when using the breakpoint method for the detection of resistance to methicillin in S. aureus. In our study, of all the media tested, Mueller-Hinton agar with 4% added NaCl incubated at 35°C gave the highest MIC's at 24 hours for the resistant isolates, confirming the findings and recommendations of Milne et al.

OXACILLIN STOKES DISC DIFFUSION METHOD

Local isolates grew poorly at 30°C after 18 hours of incubation,

making interpretation of zone sizes difficult. The growth was better after 24 hours of incubation. The "best" medium tested was Mueller-Hinton agar. On Mueller-Hinton agar none of the resistant isolates showed an intermediate susceptibility while all the other media tested yielded intermediate susceptibility results of at least some of the resistant isolates. On Mueller-Hinton agar incubated at 30°C for 18 hours, all the sensitive isolates appeared sensitive, while on Mueller-Hinton agar incubated at 35°C for 18 hours, 8 isolates appeared intermediate. The reading of Mueller-Hinton agar incubated at 35°C for 18 hours was much easier and more clearcut than when incubated at 30°C for 18 hours.

CEPHALOTHIN SUSCEPTIBILITY TESTING

Thornsberry and McDougal (22) noted that certain results may serve as "flags" to alert the laboratory worker to the possibility that the S. aureus strain is a heteroresistant one; one such indicator is cross-resistance between cephalothin and the three PRPs (methicillin, oxacillin and nafcillin). A cephalothin MIC of 2, 4 or 8 mg/l should be a clue that the strain is probably methicillin- and cephalothin-resistant.

Of the oxacillin-sensitive strains studied (microtitre MIC $\leq$ 2mg/l), 3 isolates had a cephalothin MIC =2mg/l. In this study cephalothin disc diffusion susceptibility was a poor indicator of oxacillin resistance since only 30% of the

oxacillin-resistant isolates were also resistant to cephalothin when tested by the Stokes disc diffusion method with Mueller-Hinton agar incubated at 35°C for 18 hours. One of the reasons for this apparently poor correlation could be the high concentration of the cephalothin disc (30 ug) employed.

ASSOCIATION BETWEEN OXACILLIN SUSCEPTIBILITY AND SUSCEPTIBILITY TO OTHER ANTIMICROBIAL AGENTS

The third "flag" mentioned by Thornsberry and McDougal (22) is the occurrence of multiple resistance in a strain. In this study gentamicin correlated best with oxacillin; because all the oxacillin-sensitive isolates were also gentamicin-sensitive and 80% of the oxacillin-resistant isolates were also resistant to gentamicin using the disc diffusion method.

The second best correlation was with tetracycline. Of the 60 oxacillin-sensitive strains, 59 (98%) were tetracycline-sensitive and 50% of the oxacillin resistant strains were tetracycline-resistant.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, it was found that the majority of S. aureus strains which were reported as showing intermediate susceptibilty to oxacillin, as tested in the routine laboratory using Iso-Sensitest agar, were susceptible to oxacillin according to the microtitre MIC values.

This study failed to confirm intermediate susceptibility to oxacillin in any of the strains with the reference microtitre method.

From data obtained in this study, the following can be recommended:

1. For the agar dilution method, use Mueller-Hinton agar with 4% NaCl incubated at 35°C for 24 hours, because reliable distinction between sensitive and resistant isolates was achieved using this medium.
2. The medium to be used for the Stokes disc diffusion method is Mueller-Hinton agar incubated at 35°C for 18 hours, because local isolates showed good growth on this medium at 35°C incubation, and no oxacillin-resistant isolates yielded an intermediate susceptibility. Strains yielding intermediate susceptibilities by this method can be interpreted as fully sensitive.
3. Cephalothin disc diffusion susceptibility results are a poor indicator of the susceptibility of strains to the PRP's,

whilst gentamicin and tetracycline disc diffusion susceptibilities of individual strains are useful indicators of a strain's susceptibility to oxacillin and other PRP's.

Table 1. Oxacillin and cephalothin MIC 50, MIC 90, and mean values for the microtitre assay and agar dilution methods.

				MIC 50	MIC 90	MEAN
OXACILLIN						
Microtitre method			S	1	1	0.84
			R	64	128	72
Agar dilution method						
* Agar	Inc. Temp. (C)	Inc. Time (hr)				
MHA	30	24	S	0.5	0.5	0.42
			R	64	128	100.8
MHSA	35	24	S	0.5	1	0.59
			R	64	128	104
DST	30	24	S	0.5	0.5	0.43
			R	16	64	35.2
DSTS	35	24	S	0.25	0.5	0.37
			R	8	32	18.2
COL	30	24	S	0.5	0.5	0.38
			R	32	128	75.2
COLS	35	24	S	0.5	1	0.55
			R	64	64	60
CEPHALOTHIN						
Microtitre method				0.5	16	5.41
Agar dilution method				0.5	32	10.5

* MHA, Mueller-Hinton agar; MHSA, MHA plus 4% NaCl; DST, Diagnostic sensitivity test agar; DSTS, DST plus 4% NaCl; COL, Columbia agar; COLS, COL plus 4% NaCl.

Table II. Comparison between oxacillin microtitre assay and disc diffusion method using Mueller-Hinton agar.

MIC (mg/l)			0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	>128.0
No. of isolates			1	4	13	35	7	0	0	2	2	13	2	1
* Agar	Inc. Temp (°C)	Inc. Time (hr)	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
MHA	30	18	1	4	13	35	7			2	2	13	2	1
		24	1	4	13	34 1	6 1			2	2	13	2	1
		48	1	4	13	29 6	4 3			2	2	13	2	1
MHA	35	18	1	4	11 2	31 4	5 2			2	2	13	2	1
		24	1	4	11 2	26 9	5 2			2	2	13	2	1
		48	1	3 1	10 2 1	24 10 1	3 3 1			2	2	13	2	1
MHSA	35	18	1	4	11 2	20 15	5 2			2	2	13	2	1
		24	1	4	8 5	12 23	4 3			2	2	13	2	1
		48	1	2 2	7 5 1	3 14 18	2 5			2	2	13	2	1

* MHA, Mueller-Hinton agar; MHSA, MHA plus 4% NaCl.

Table III. Comparison between oxacillin microtitre assay and disc diffusion method using Diagnostic sensitivity test agar.

MIC (mg/l)			0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	>128.0
No. of isolates			1	4	13	35	7	0	0	2	2	13	2	1
* Agar	Inc. Temp (°C)	Inc. Time (hr)	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
DST	30	18	1	4	13	30 5	6 1			2	2	13	2	1
		24	1	4	13	21 14	5 2			2	2	13	2	1
		48	1	3 1	12 1	14 19 2	2 4 1			2	2	13	2	1
DST	35	18	1	4	10 3	24 11	4 3			2	2	2 11	2	1
		24	1	4	9 4	13 22	4 3			2	1 1	1 12	2	1
		48	1	4	8 5	8 25 2	1 6			2	2	13	2	1
DSTS	35	18	1	4	10 3	22 13	4 3			1 1	1 1	3 10	2	1
		24	1	4	8 5	12 23	2 5			1 1	2	2 11	1 1	1
		48	1	3 1	6 6 1	25 10	5 2			1 1	2	1 12	2	1

* DST, Diagnostic sensitivity test agar; DSTS, DST plus 4% NaCl.

Table IV. Comparison between oxacillin microtitre assay and disc diffusion method using Columbia agar.

MIC (mg/l)			0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	>128.0
No. of isolates			1	4	13	35	7	0	0	2	2	13	2	1
* Inc. Agar	Inc. Temp (°C)	Inc. Time (hr)	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
COL	30	18	1	4	13	33 2	7			1 1	2	13	2	1
		24	1	3 1	13	29 6	5 2			1 1	2	13	2	1
		48	1	3 1	12 1	22 11 2	2 5			2	2	13	2	1
COL	35	18	1	4	13	25 10	5 2			2	2	13	2	1
		24	1	4	11 2	21 14	4 3			2	2	13	2	1
		48	1	4	8 5	18 17	2 5			1 1	2	13	2	1
COLS	35	18	1	4	10 3	15 20	4 3			2	2	1 12	2	1
		24	1	4	9 4	10 25	2 5			2	2	13	2	1
		48	1	3 1	8 5	1 18 16	1 3 3			2	2	13	2	1

* COL, Columbia agar; COLS, COL plus 4% NaCl.

TABLE V. Comparison between oxacillin microtitre assay and disc diffusion method using Iso sensitest agar.

MIC (mg/l)			0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	>128.0
No. of isolates			1	4	13	35	7	0	0	2	2	13	2	1
* Inc. Agar	Inc. Temp (°C)	Inc. Time (hr)	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
ISO	30	18	1	4	12 1	24 11	5 2			2	1 1	1 12	2	1
		24	1	4	12 1	18 17	4 3			2	1 1	13	2	1
		48	1	4	9 4	8 27	2 5			2	2	13	2	1

* ISO, Iso sensitest agar.

TABLE VI. Comparison between oxacillin and cephalothin minimum inhibitory concentrations.

Oxacillin MIC (mg/l)	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	>128.0
Cephalothin MIC (mg/l)												
≤1	1	4	13	33	6							
2				2	1							
4									1			
8									1	2		
16								2		8	1	
≥32										3	1	1

Table VII.Comparison between cephalothin microtitre assay and disc diffusion method.

MIC (mg/l)	<0.12	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0
No. of isolates	1	0	16	25	15	3	1	3	11	4	1
* Agar / Inc. Temp (°C) / Inc. Time (hr)	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R	S I R
MHA 35 18	1		15 1	23 2	14 1	2 1	1	3	7 4	3 1	1

* MHA, Mueller-Hinton agar.

Table VIII. The association between oxacillin susceptibility (according to MIC) and susceptibility to other antimicrobial agents, as determined by Stokes disc diffusion method with MHA, incubated at 35°C for 18 hours.

Oxacillin MIC (mg/l)		2	8
Total number of isolates		60	20
Penicillin	Sensitive	8	0
	Intermediate	46	1
	Resistant	6	19
Erythromycin	Sensitive	56	9
	Intermediate	0	5
	Resistant	4	6
Tetracycline	Sensitive	59	7
	Intermediate	0	3
	Resistant	1	10
Oxacillin	Sensitive	52	0
	Intermediate	8	0
	Resistant	0	20
Chloramphenicol	Sensitive	59	16
	Intermediate	0	0
	Resistant	1	4
Cephalothin	Sensitive	55	2
	Intermediate	5	12
	Resistant	0	6
Clindamicin	Sensitive	60	19
	Intermediate	0	0
	Resistant	0	1
Gentamicin	Sensitive	60	2
	Intermediate	0	2
	Resistant	0	16

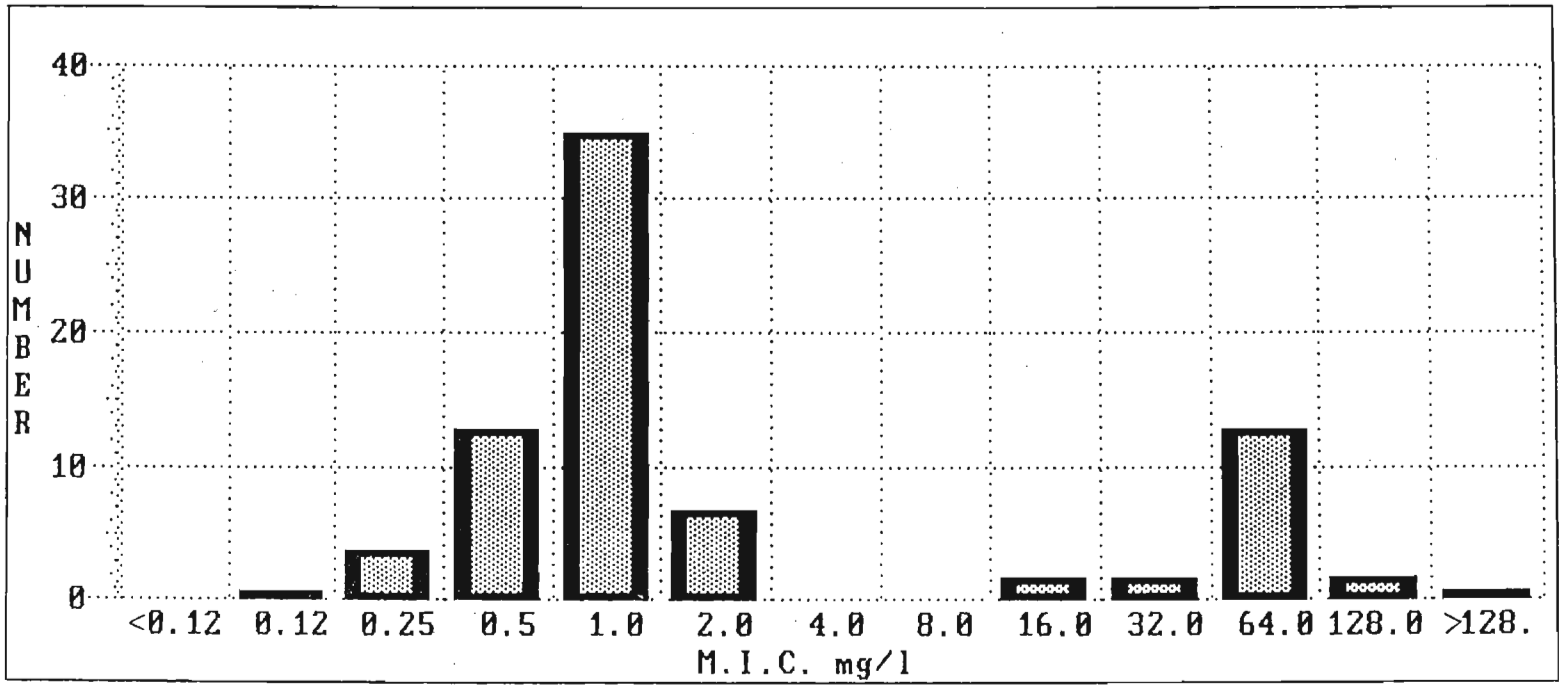

FIGURE 1 : Distribution of isolates according to oxacillin microtitre assay

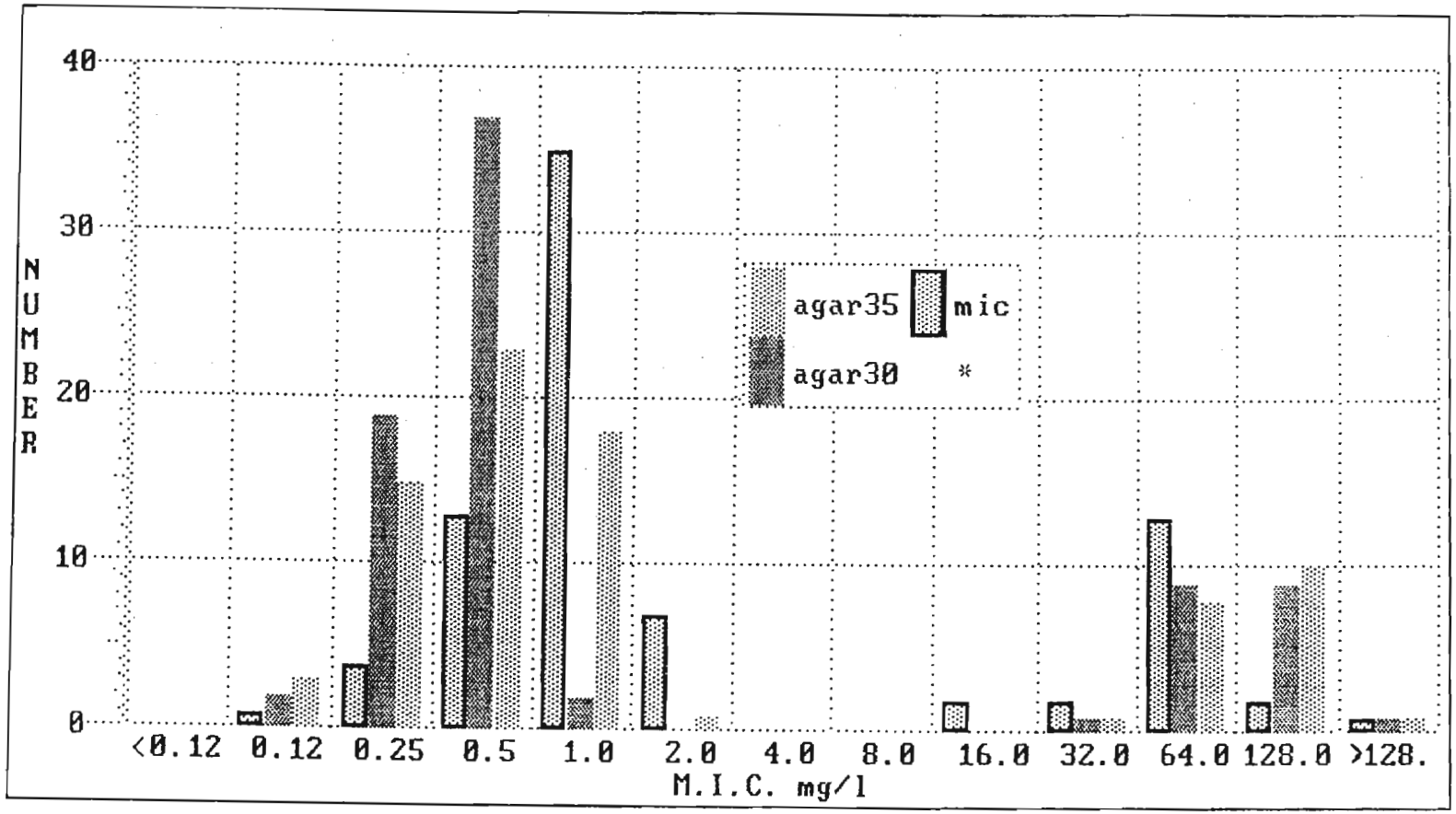

FIGURE 2 : Comparision between oxacillin microtitre assay and Mueller-Hinton agar dilution method.

*Agar 35, Mueller-Hinton agar with 4% NaCI incubated at 35°C for 24 hours; Agar 30, Mueller-Hinton agar incubated at 30°C for 24h; MIC, microtitre method.

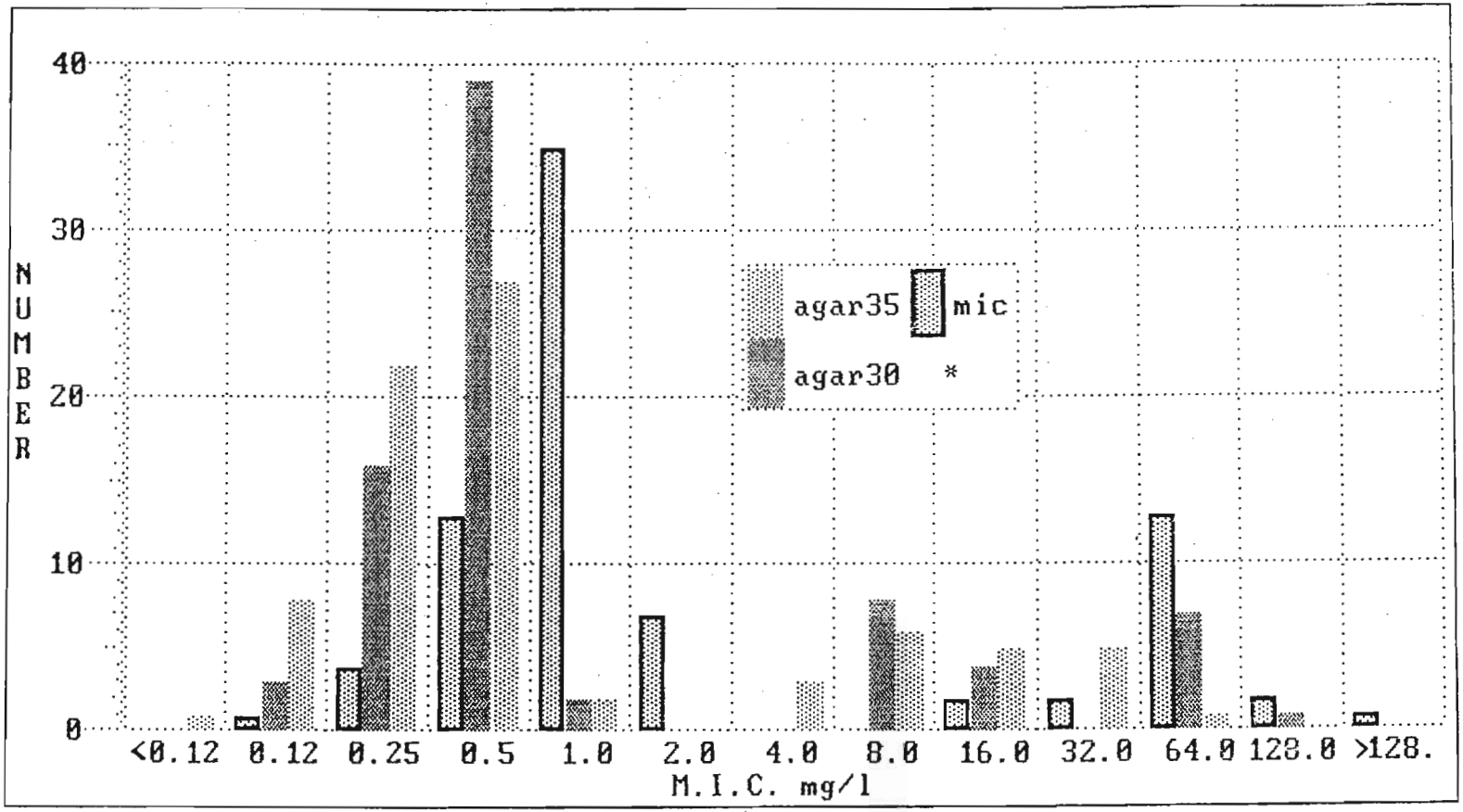

FIGURE 3 : Comparision between oxacillin microtitre assay and Diagnostic sensitivity test agar dilution method.

*Agar 35, Diagnostic sensitivity test agar with 4% NaCI incubated at 35°C for 24 hours; Agar 30, Daignostic sensitivity test agar incuated at 30°C for 24h; MIC, microtitre method.

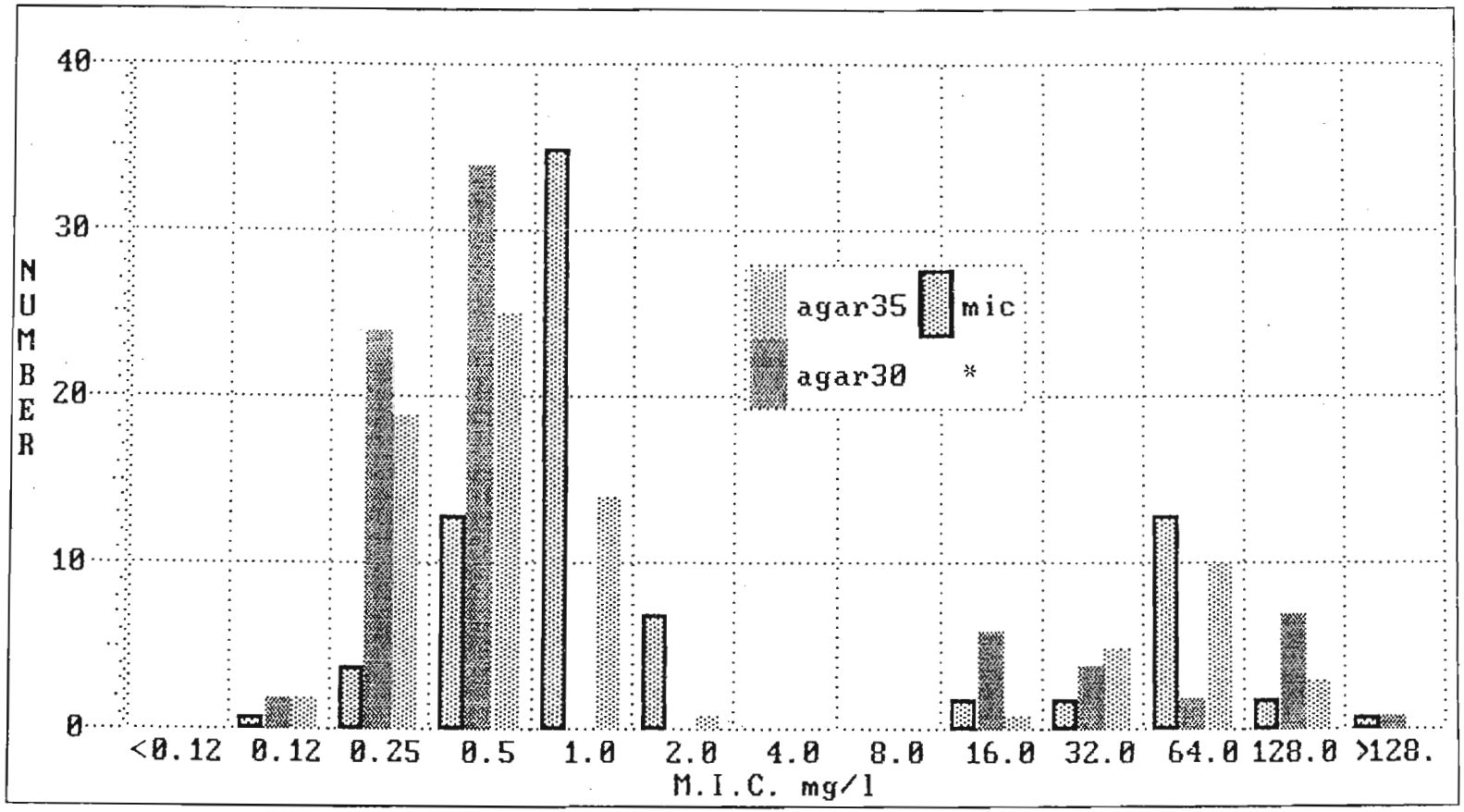

FIGURE 4 : Comparison between oxacillin microtitre assay and Columbia agar dilution method.

*Agar 35, Columbia agar with 4% NaCl incubated at 35°C for 24hours ; Agar 30, Columbia agar incubated at 30°C for 24h; MIC, microtitre method.

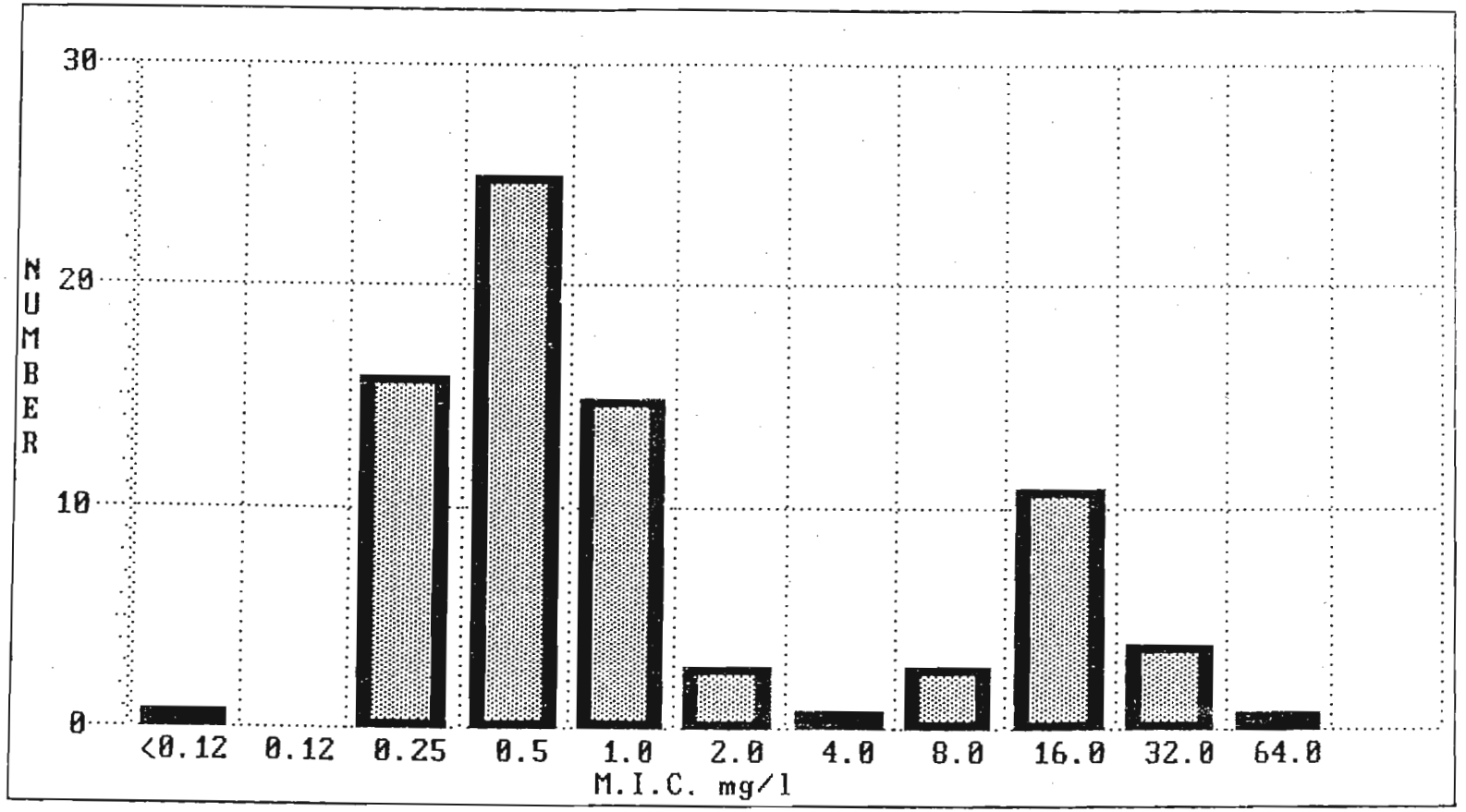

FIGURE 5 : Distribution of isolates according to cephalothin microtitre assay.

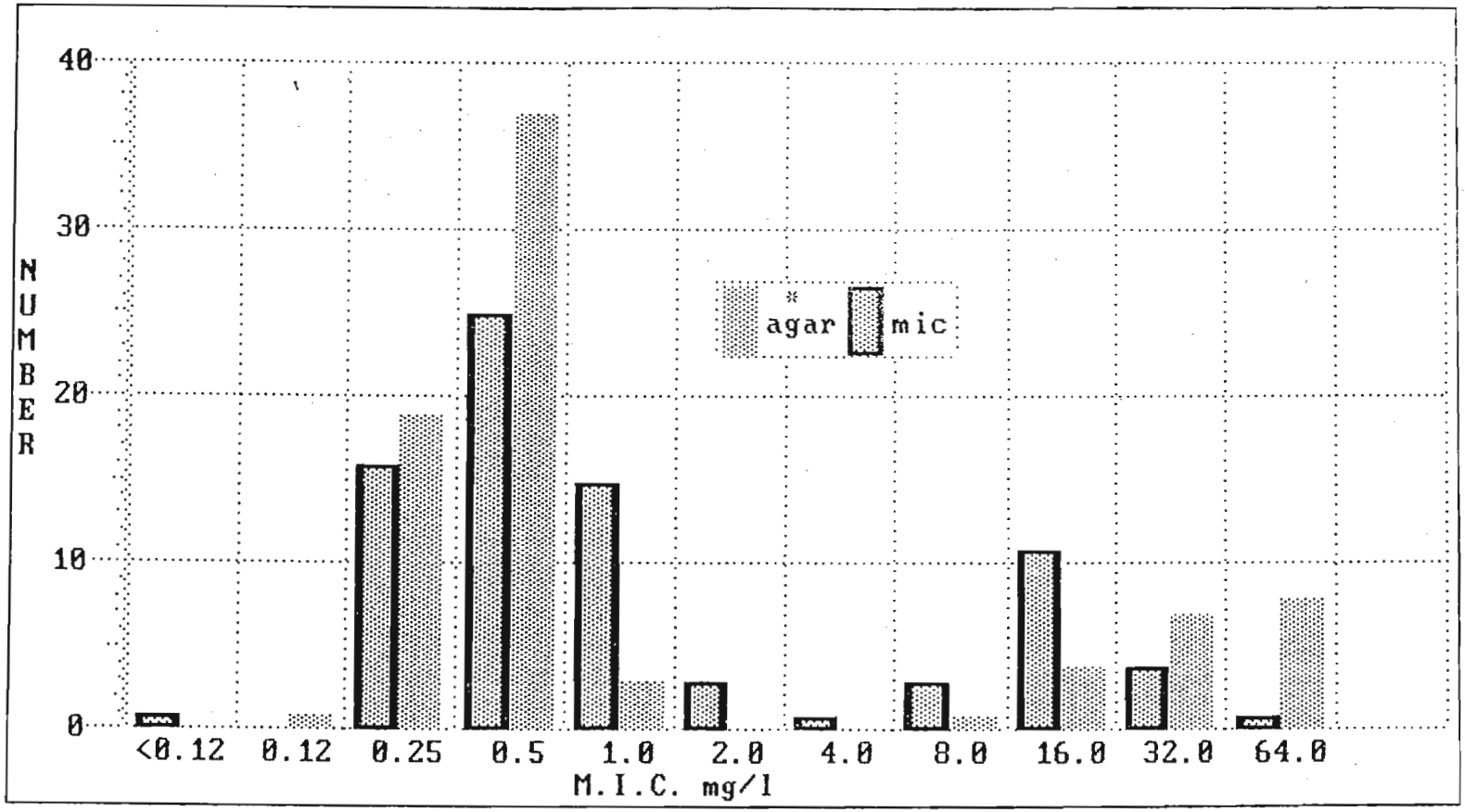

FIGURE 6 : Comparision between cephalothin microtitre assay and Mueller-Hinton agar dilution method.

*Agar, Mueller-Hinton agar with 4% NaCI incubated at 35°C for 24 hours; MIC, microtitre method.

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